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LIQUID CHROMATOGRAPHIC ASSAY OF THEBAINE IN PAPAVER BRACTEATUM ^{2/}

by: W.B. Caldwell, ^{2/}

Department of Analytical and Physical Chemistry,
Merck and Co. Inc.,
Rahway, N.J. 07065, United States of America.

- ^{1/} General note: The views expressed by collaborating scientists in their contributions to the programme are not necessarily shared by the Secretariat.
- ^{2/} In the report of the Second Working Group on Papaver bracteatum (document ST/SOA/SER.J/2), it was recommended that methods for the determination of thebaine in Papaver bracteatum should be compared and evaluated; to achieve this, it was decided that participants in the Working Group would forward their methods for publication in this series so that these could be tried out in the various laboratories.
- ^{3/} This paper was submitted by Dr. E.F. Schoenewaldt, Manager, Process Research, Merck and Co. Inc.

Introduction

A simple assay procedure featuring a methanolic-ammonia extraction and a high pressure liquid chromatographic separation (cation exchange) is described for thebaine in Papaver bracteatum. The results of this procedure for three P. bracteatum samples obtained from Dr. Phillip Vincent of the United States Department of Agriculture are shown in Table I. These samples were assayed as received. The effects of sample particle size distribution, extraction efficiency and stability of thebaine on the precision of the assay are discussed.

Experimental

A. Apperatus

1. Liquid chromatograph - Du Pont Model 840.
2. 25 microliter syringe - Hamilton 702.
3. 15 milliliter centrifuge tubes fitted with polyethylene caps.
4. Ultrasonic bath.

B. Chemicals

1. Boric acid.
2. 50% Sodium hydroxide.
3. Potassium nitrate.
4. Acetonitrile - spectroquality.
5. n-Propanol - spectroquality.
6. Methanol.
7. Ammonium hydroxide.

C. Solutions

1. Extractant - 98/2 (v/v) methanol/ammonium hydroxide.
2. Chromatographic eluent - 0.3M NaOH adjusted to pH 9.5 with boric acid, 0.2M KNO₃, 4% acetonitrile, 1% n-propanol.
 - (a) Preparation of 4 liters of the 0.3M NaOH solution:

50% Sodium hydroxide	- 60 ml
Boric acid	- 114 g (approximate)
Distilled water	- 4000ml

(b) Preparation of 4 liters of the chromatographic eluent:

3800 ml - Solution from Step (a)
80.9 g - Potassium nitrate
160 ml - Acetonitrile
40 ml - n-Propanol

(c) Degas the solution prepared in Step (b) by bubbling helium through the solution for 5-10 minutes.

D. Chromatographic Conditions

Column - 1 m x 2.1 mm (id) strong cation exchange, Du Pont SCX.

Eluent - 0.03M NaOH adjusted to pH 9.5 with boric acid,
0.2 M KNO_3 , 4% acetonitrile, 1% n-propanol.

Pressure - 1200 psig.

Flow - 0.8 ml/min.

Temperature - ambient.

Detection - UV₂₅₄ nm, attenuation 0.02 AUF.

Chart Speed - 0.2 in/min.

Injection Size - 10.0 microliters.

E. Thebaine Standard - 0.400 mg/ml

Weigh accurately 10 mg of the thebaine reference material into a 25 ml volumetric flask and dilute to the mark with 98/2 (v/v) methanol/ammonia.

The standard solution is stable for at least a week if refrigerated.

F. Assay Procedure

1. Weigh accurately 200 mg of the sample into a 15 ml centrifuge tube.
2. Accurately pipette 3 ml of the 98/2 (v/v) methanolic/ammonia extractant into the tube if the sample is roots or shoots and 10 ml if the sample is a capsule.
3. Cap the tube with a polyethylene cap and shake continuously for 20 minutes with occasional ultrasonification.
4. Centrifuge the sample for 5 minutes to clear the liquid layer.
5. Inject duplicate aliquots of exactly 10 microliters of the sample solution (liquid layer - Step 4) into the liquid chromatograph.

6. Inject duplicate aliquots of exactly 10 microliters of the thebaine standard solution into the liquid chromatograph.
7. Calculate the Wt % of thebaine in the sample by the following relationship:

$$\text{Wt \% thebaine} = \frac{A_X \cdot C_S \cdot D \cdot 100}{A_S \cdot W_X}$$

where A_X = Sample thebaine peak area.

A_S = Standard thebaine peak area.

C_S = Concentration of the thebaine standard.

W_X = Weight of the thebaine sample.

D = Milliliters of extractant.

example - Capsule (*P. bracteatum*, Arya II from Iran, 12/73)

$$A_X = 2.77 \text{ in}^2 \quad [\text{peak area} = \text{h} \cdot \text{w} \text{ (at } 1/2 \text{ h)}]$$

$$A_S = 2.75 \text{ in}^2$$

$$C_S = 0.507 \text{ mg/ml}$$

$$W_X = 178.5 \text{ mg}$$

$$D = 10$$

$$\text{Wt \% thebaine} = \frac{2.77 \cdot 0.507 \cdot 10 \cdot 100}{2.75 \cdot 178.5} = 2.86$$

Results and Discussion

The results of the thebaine assay for three samples sent to Dr. Schoenewaldt by Dr. Vincent of the USDA are listed in Table I, and their liquid chromatograms are shown in Figures 1, 2 and 3. The USDA assays on the root and shoot samples are reported to be 0.4% and on the capsule to be 4.3%. Our liquid chromatographic assays on these samples are 30-50% lower. Three factors were investigated to account for these discrepancies.

1. Thebaine solvent stability - The assay of a root extract did not significantly change after 5 days of aging the extract at room temperature. The solvent was the 98/2 (v/v) methanolic/ammonia extractant.

2. Extraction efficiency - A root sample was sampled after 20 minutes and 90 minutes of extraction. The assays were not significantly different.
3. Sample particle size distribution - An aliquot (2.9 g) of the capsule sample (ground to 40 mesh) was dry sieved for 20 minutes into four fractions and each fraction then assayed for thebaine. The results of these experiments are listed in Table II. The data clearly show that the 40 mesh sample is inhomogeneous with respect to thebaine content because the smaller particle fractions contained more thebaine than did the bulk sample. The amounts of thebaine obtained from the weighted sum of the four fractions and from the bulk sample were not significantly different (87.3 mg vs 86.0 mg) suggesting that the solvent extraction was complete and that the bulk sample had not yet suffered much particle size segregation (i.e. smaller particles settling to the bottom of the vial). An additional experiment was performed on the 100 mesh fraction material. This material was ground by mortar and pestle and then separated into two fractions with a 100 mesh sieve. The amounts of thebaine obtained from the weighted sum of the two fractions, and the original 100 mesh material were not significantly different as shown in Table III. If the weighted sum assays had been significantly larger than the bulk assays, then one could have assumed that the smaller particle material was extracted more efficiently than the larger particle material. The data do not support this argument. These experiments clearly show that the solvent extraction is complete and suggest that there are fines rich in thebaine which adhere to the larger sample particles. Experiments should be performed to determine the optimum sample size, sieve fraction and sieving time to produce an accurate assay for thebaine in the various parts of the plant.

Conclusion

The liquid chromatographic assay for thebaine is a simple procedure requiring few laboratory steps. However, the precision of the assay and inter-laboratory assay correlation will depend on a well defined sampling procedure. It is apparent from the data in Table II that sampling 40 mesh ground plant material could lead to erroneous results.

Table I

Liquid chromatographic assay of the aine in various P. bracteatum samples

<u>Sample</u>	<u>Sample weight</u>	<u>Extractant Volume</u>	<u>Wt. % thebaine</u>
Roots (UNB 4)	190.5 mg	3 ml	0.22
Shoots (UNB 4)	173.5 mg	3 ml	0.08
Capsule (Arya II from Iran, 12/73)	178.5 mg	10 ml	2.86

Table II

Thebaine content of various particle size fractions
of the P. bracteatum 40 mesh capsule sample

<u>Mesh size</u>	<u>Sieve Wt</u>	<u>Data %</u>	<u>Wt of thebaine</u>	<u>Wt % thebaine</u>
> 40 (420 μ)	2.90 g	100%	86.0 mg	2.97
< 100 (149 μ)	1.37 g	47.2	27.3 mg	1.99
100-200 (149 μ - 74 μ)	.47 g	16.2	18.9 mg	3.40
200-325 (74 μ - 44 μ)	.80 g	27.6	29.5 mg	3.69
> 325 (44 μ)	.26 g	9.0	11.6 mg	4.46
		100.0	87.3 mg	

Table III

Thebaine content of the ground 100 mesh fraction of
the P. bracteatum capsule sample

<u>Mesh size</u>	<u>Sieve Wt</u>	<u>Data %</u>	<u>Wt of thebaine</u>	<u>Wt % thebaine</u>
> 100	1.22 g	100%	24.2 mg	1.99
> 100	.78 g	64%	11.2 mg	1.44
< 100	.44 g	36%	12.4 mg	2.81
		100%	23.6 mg	

Figure 1. Roots, P. bracteatum

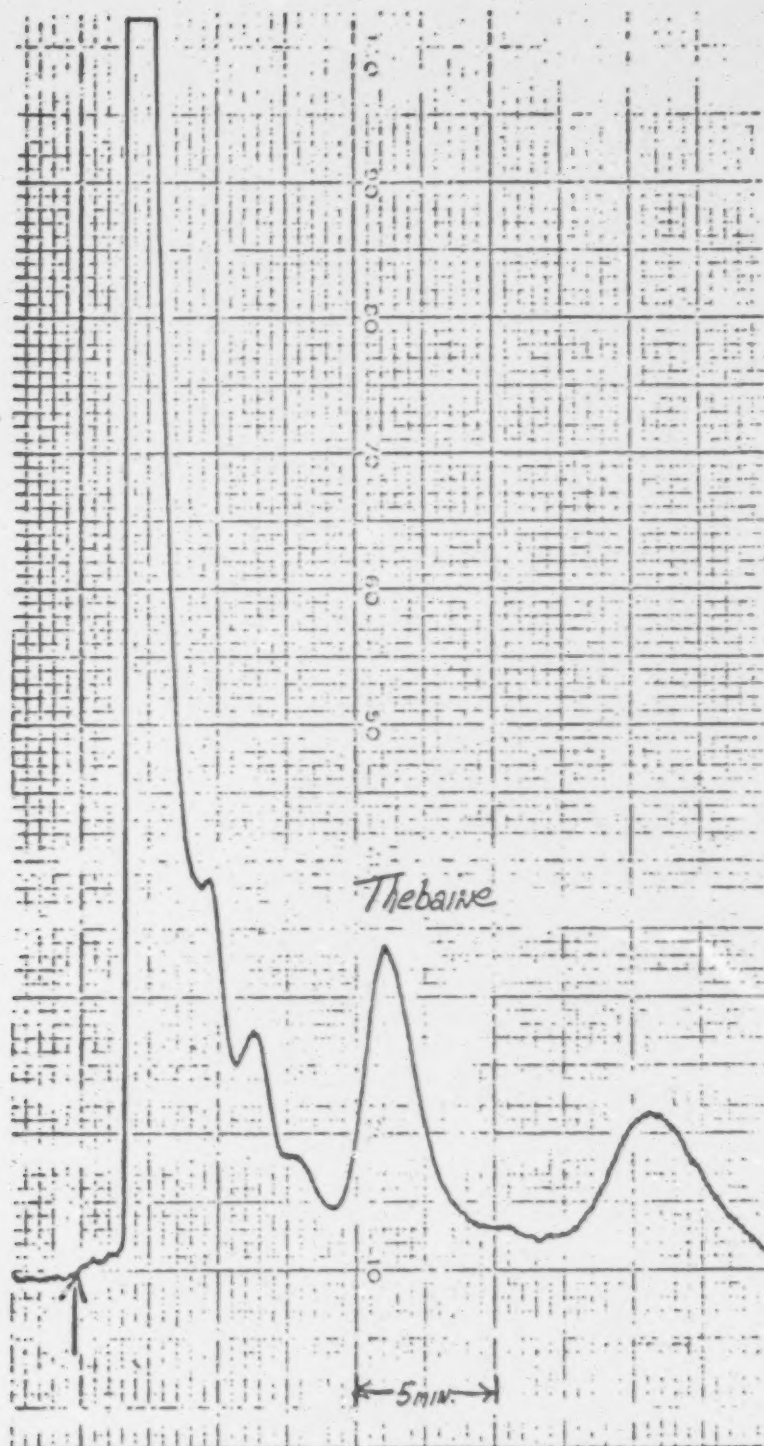


Figure 2. Shoots, *P. bracteatum*

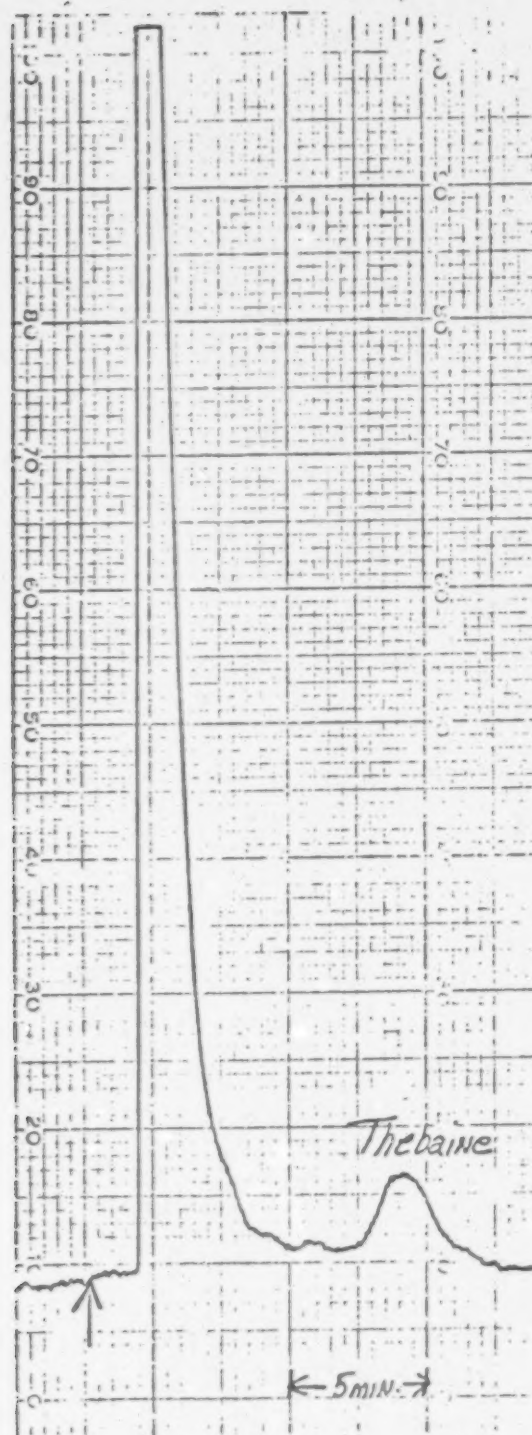


Figure 3. Capsule, P. bracteatum

